Biochemical and Biophysical Research Communications 470 (2016) 239–244

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Development of an ErbB4 monoclonal antibody that blocks neuregulin-1-induced ErbB4 activation in cancer cells

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ARTICLE INFO

Article history:
Received 5 January 2016
Accepted 8 January 2016
Available online 11 January 2016

Keywords:
ErbB4
Neuregulin
Neutralizing antibody
Breast cancer
Three-dimensional culture

ABSTRACT

The use of monoclonal antibodies (mAbs) for cancer therapy is one of the most important strategies for current cancer treatment. The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, which regulates cancer cell proliferation, survival, and migration, is a major molecular target for antibody-based therapy. ErbB4/HER4, which contains a ligand-binding extracellular region, is activated by several ligands, including neuregulins (NRGs), heparin-binding EGF-like growth factor, betacellulin and epiregulin. Although there are clinically approved antibodies for ErbB1 and ErbB2, there are no available therapeutic mAbs for ErbB4, and it is not known whether ErbB4 is a useful target for antibody-based cancer therapy. In this study, we developed an anti-ErbB4 mAb (clone P6-1) that suppresses NRG-dependent activation of ErbB4 and examined its effect on breast cancer cell proliferation in the extracellular matrix.

1. Introduction

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases consists of ErbB1 (also known as EGFR and HER1), ErbB2 (c-Neu, HER2), ErbB3 (HER3), and ErbB4 (HER4). The activation of the ErbB receptors stimulates intracellular signaling pathways, including the RAS-RAF-MEK-ERK, PI3K-AKT, and JAK-STAT pathways, that promote cell proliferation, survival, and migration. Therefore, aberrant activation of ErbB receptors is frequently observed in multiple types of human cancers [1].

ErbB4 is a 180 kDa glycoprotein consisting of a ligand-binding extracellular region, a transmembrane region, and a cytoplasmic region containing a tyrosine kinase domain. ErbB4 is activated by the ligands neuregulin 1-4 (NRG1-4) [2–5], heparin-binding EGF (HB-EGF) [6], betacellulin [7], and epiregulin [8], and forms homo- or heterodimers with other EGFR family proteins. Recently, ErbB4 was shown to play an essential role in the development and maintenance of the heart, mammary glands, and nervous system [9–12]. Expression of ErbB4 affects the growth of human breast cancer cells [13], and it transforms mouse mammary epithelial cells *in vitro* and *in vivo* [14]. On the other hand, stimulation of breast cancer cells with NRG-1 suppresses cell growth in an ErbB4-dependent manner [15,16]. Thus, little is known about the functional relevance of ErbB4 to the behavior of cancer cells.

The use of monoclonal antibodies (mAbs) to block oncogenic receptors is an established mode of cancer therapy [17]. However, despite the clinical success of ErbB1 and ErbB2 antibodies, it is unknown whether ErbB4 is a useful molecular target for antibody-based cancer therapy. To examine this possibility, we established a novel human ErbB4 antibody that blocks NRG-1-ErbB4 signaling and examined its effect on cancer cells.

2. Results

2.1. Generation of human ErbB4-specific mAbs

To examine the functional role of ErbB4 in cancer cells, a mAb against human ErbB4 (anti-ErbB4 mAb) was generated. First, rat hepatoma RH7777 cells stably expressing GFP-tagged ErbB4 (ErbB4-GFP) were generated; then, F344/N rats were immunized with the cells several times. After confirmation of a raised antibody titer in immunized rat serum, spleen-derived lymphocytes were

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http://dx.doi.org/10.1016/j.bbrc.2016.01.045

fused with X63 or P3U1 mouse myeloma cells, and four hybridoma cell lines secreting ErbB4 mAbs were obtained (Fig. 1A).

To analyze the specificity of the anti-ErbB4 mAbs, their reactivity to GFP-tagged ErbB1-, ErbB2-, ErbB3-, or ErbB4-expressing RH7777 cells was evaluated by flow cytometry. The anti-ErbB4 mAbs (P6-1, P32-1, 1–3C, and 3–10A) were found to bind strongly to the ErbB4-expressing RH7777 cells in a GFP expression level-dependent manner, and had low or negligible reactivity to the other ErbB family members (Fig. 1B). These results indicate that the four newly developed anti-ErbB4 mAbs are specific for the human ErbB4 protein.

2.2. ErbB4 expression analysis on various cancer cell lines

Flow cytometry was next performed to examine the reactivity of the four anti-ErbB4 mAbs to various human cancer cell lines. Using the clone P6-1, which exhibited the strongest reactivity to ErbB4, we found that ErbB4 was highly expressed on breast cancer cell lines compared with other types of cancer cells (Fig. 2). As previously reported [18], the estrogen receptor-positive breast cancer cell lines T47D and MCF7 were found to have the higher ErbB4 expression among the breast cancer cell lines (Fig. 2). Furthermore, esophageal cancer (TE1), bladder cancer (KU-1), lymphoma (Raji), and embryonic kidney (HEK293F) cells also expressed high levels of ErbB4 (Fig. 2). These results indicate that ErbB4 is differentially expressed in human cancer cell lines.

2.3. Inhibition of ligand-dependent ErbB4 phosphorylation with anti-ERBB4 mAbs

Given that the development of blocking antibodies is a therapeutic strategy for cancer treatment, we examined whether the newly developed ErbB4 mAbs are able to inhibit ligand-dependent ErbB4 phosphorylation (activation) in cancer cells. Addition of neuregulin-1 (NRG-1), a known ligand for ErbB4, to the culture medium strongly induced ErbB4 phosphorylation in T47D cells, which expressed high levels of ErbB4 (Fig. 3A), suggesting that NRG-1 acts as a ligand for ErbB4 in these cells. Pre-treatment of the cells with the anti-ErbB4 mAb P6-1, but not P32-1, 1–3C, or 3–10A, significantly reduced the NRG-1-dependent phosphorylation of ErbB4 in T47D cells (Fig. 3A), suggesting that P6-1 may block NRG-1-induced ErbB4 signaling.

To further examine whether P6-1 acts as a neutralizing antibody for ErbB4, the effect of NRG-1 was examined on another breast cancer cell line, MCF7. Treatment with P6-1 strongly suppressed the NRG-1-induced activation of ErbB4 in a concentration-dependent manner (Fig. 3B). These results suggest that P6-1 acts as a neutralizing antibody for ErbB4 in cancer cells.

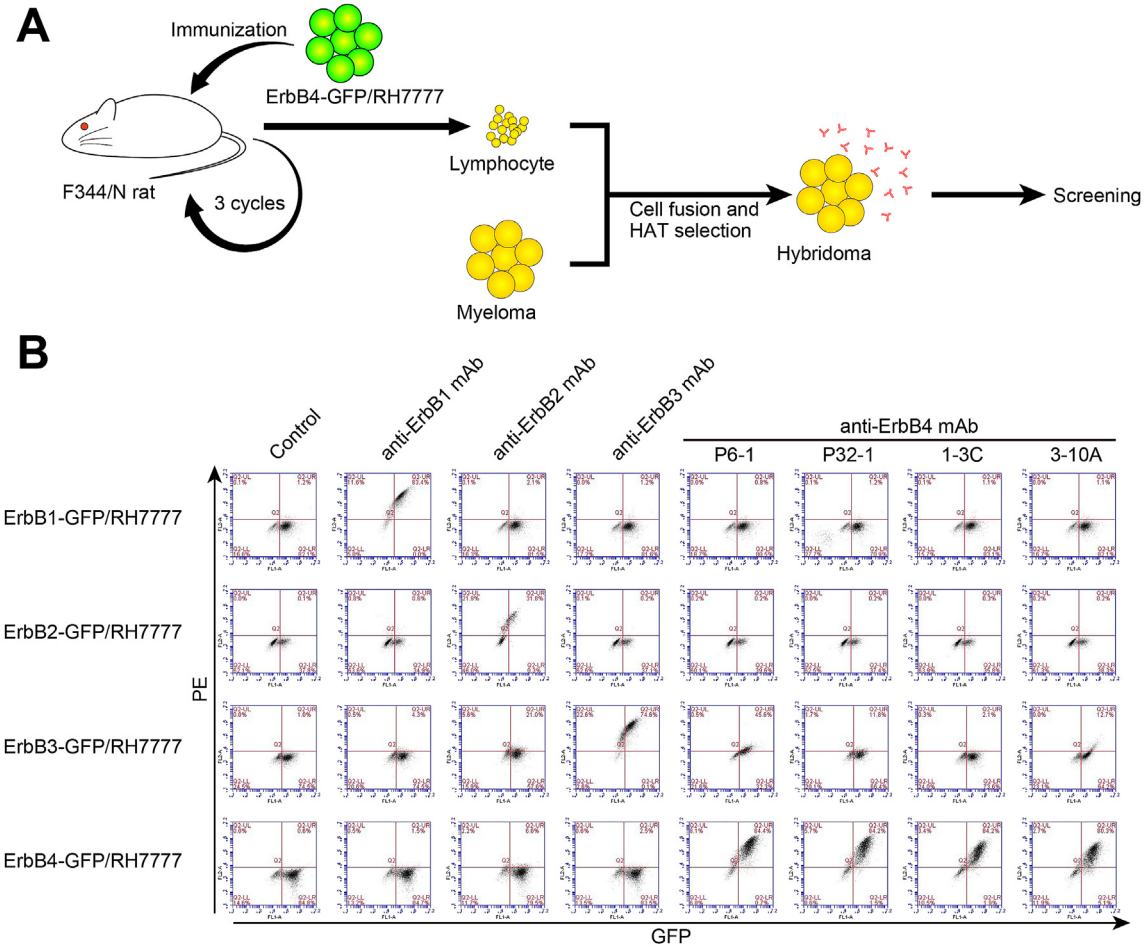

Fig. 1. Development of ErbB4-specific monoclonal antibodies. A. Schema for immunization and hybridoma development. B. Stably GFP-tagged ErbB1-, ErbB2-, ErbB3-, or ErbB4-expressing RH7777 cells were incubated with indicated mAbs followed by the staining with PE-conjugated anti-rat IgG secondary antibodies and were subjected to flow cytometric analysis. PE and GFP fluorescence intensities are plotted on the vertical and horizontal axes, respectively. Note that anti-ErbB1, ErbB2 and ErbB3 mAbs specifically reacted with ErbB1, ErbB2 and ErbB3-expressing RH7777 cells respectively, whereas anti-ErbB4 mAbs specifically reacted with ErbB4- expressing RH7777 cells.